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Pre-extraction Preparation (Fresh, Frozen, Freeze-Dried, or Acetone Powdered) and Long-Term Storage of Fruit and Vegetable Tissues: Effects on Antioxidant Enzyme Activity

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Activities of the antioxidant enzymes ascorbate peroxidase, catalase, dehydroascorbate reductase, glutathione reductase, guaiacol peroxidase, monodehydroascorbate reductase, and superoxide dismutase were assayed in honeydew (*Cucumis melo* L.) fruit and spinach (*Spinacia oleracea* L.) leaves either as fresh, frozen to −80 °C, frozen in liquid nitrogen, freeze-dried, or acetone powder, representing the various ways tissues are treated prior to enzyme extraction. Treated tissues were analyzed following treatment or stored for up to 8 weeks at −80 °C. Enzyme activities in fruit frozen with or without liquid nitrogen and leaves frozen with or without liquid nitrogen or freeze-dried were equal to those of fresh tissue. Enzyme activities in freeze-dried or acetone-powdered fruit and leaves and in acetone-powdered tissues were significantly higher or lower than those in fresh tissue. Enzyme activities in both tissues frozen with or without liquid nitrogen and stored for 8 weeks at −80 °C changed little; those in freeze-dried and acetone-powdered tissues, however, significantly increased/decreased over the same period. Fresh tissue should be used in antioxidant enzyme assays, but if storage is necessary, tissues should be placed directly into a −80 °C freezer.

KEYWORDS: Chenopodiaceae; Cucurbitaceae; *Cucumis melo* L. inodorus group); ascorbate peroxidase; catalase; dehydroascorbate reductase; glutathione reductase; guaiacol peroxidase; monodehydroascorbate reductase; superoxide dismutase

INTRODUCTION

Increasing focus is being given to enzymic and nonenzymic antioxidants in relation to storage, shelf life, and nutritional quality of postharvest fruits and vegetables (*1*−*6*). The antioxidant enzymes ascorbate peroxidase (AsPX; EC 1.11.1.11), catalase (CAT; EC 1.11.1.6), dehydroascorbate reductase (DHAR; EC 1.8.5.4), glutathione reductase (GR; EC 1.6.4.2), guaiacol peroxidase (POX; EC 1.11.1.7), monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), and superoxide dismutase (SOD; EC 1.15.1.1), found in almost all common fruits and vegetables, either are responsible for reducing the active oxygen species superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) or are directly responsible for recycling ascorbic acid and/or glutathione (*3*). Fruits and vegetables utilize these enzymatic antioxidants in various combinations to regulate and maintain active oxygen species (AOS) at controlled steady-state concentrations: SOD dismutates $O_2^{\bullet -}$, CAT, AsPX, and POX catabolize H_2O_2, GR reduces glutathione, and DHAR and MDHAR reduce ascorbate (vitamin C) (*6*).

Extraction and activity determination of antioxidant enzymes from fruits and vegetables primarily follow well-documented procedures, whereas pre-extraction preparation of plant tissues does not. A review of the literature indicates that pre-extraction tissue preparation can entail fresh (*1*), frozen (*2*), freeze-dried (*7*), or acetone-precipitated protein powder protocols (*8*).

Currently no study exists wherein enzyme activities are compared following various pre-extraction preparations of fruit and vegetable tissues. The purpose of this study is to compare and contrast a representative fruit [*Cucumis melo* L. inodorus group (honeydew melon)] and vegetable [*Spinacia oleracea* L. (spinach)] for differences in AsPX, CAT, DHAR, GR, MDHAR, POX, and SOD enzymic activities either immediately following pre-extraction preparation of tissues as (i) fresh, (ii) frozen at −80 °C, (iii) flash-frozen in liquid N_2, (iv) freeze-dried, and (v) acetone-precipitated protein powder or following 2, 4, or 8 weeks storage of ii−v at −80 °C.

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MATERIALS AND METHODS

Plant Material. Non-netted muskmelons (*Cucumis melo* inodorus group; honeydew) with a smooth epidermis and green, edible flesh (mesocarp) were obtained from a local supermarket (HEB, Weslaco, TX) on February 4 (set 1) and 11 (set 2), 2003. Spinach (*Spinacia oleracea* L.) seeds (BJ 412 Sponsor, Bejo Seeds, Warmenhuizen, The Netherlands) were sown 1.5 cm deep in potting soil (3 peatmoss/2 perlite/2 sand) in 60-cm^3 standard pots and placed into growth chambers (Econaire GR36; Econaire; Winnipeg, MB, Canada) maintained at 18 °C and 95% relative humidity with a photocycle of 10:14 L/D. The lamps (cool-white fluorescent) provided a photosynthetic photon flux of 400−450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 15 cm above soil level (LI-188B quantum sensor; Li-Cor, Lincoln, NE). Plants were routinely watered and fertilized twice weekly with water-soluble 15N/6.6P/14.8K (Plant Products, Brampton, ON, Canada) as per directions. Forty pots of spinach were grown for either 73 days (set 1) or 42 days (set 2).

Preparation of Honeydew Mesocarp Tissue. All fruits were chilled to 4 °C and washed with distilled water, the epidermis was removed with a vegetable peeler, and the polar ends (totaling two-thirds of the fruit) were removed and discarded. Wedges of the remaining equatorial region mesocarp tissue, devoid of seeds and integument tissue, from five fruits were pureed in a food processor (Quick 'N Easy, Black & Decker, Towson, MD) using 3−5 s pulses. Fifteen grams of fresh pureed tissue was either analyzed immediately (fresh) or were weighed into 50-mL Nalgene tubes, capped, and either immersed in liquid nitrogen until solid (frozen + N_2), frozen at −80 °C in a Revco Ultra cold freezer (Revco Scientific Inc., Asheville, NC) (frozen), freeze-dried after freezing to −80 °C (Virtis Freezemobile, The Virtis Co., Inc., Gardiner, NY) (freeze-dried), or reduced to an acetone-precipitated protein powder. Acetone-precipitated protein powders were prepared by adjusting the homogenate supernatant to 80% acetone with 4 °C 100% acetone (acetone ppt). The solution was placed on ice until precipitates formed and centrifuged at 10000g_n for 15 min at 4 °C, and the pellet was washed with 100% acetone and then dried at room temperature with N_2. Tissues were either assayed immediately following preparations or stored for 2, 4, or 8 weeks at −80 °C prior to analysis.

Preparation of Spinach Leaves. Spinach leaves were harvested, deribbed, and chopped into 1-cm^2 pieces. Pieces were well-mixed and subsamples immediately analyzed for antioxidant enzyme activities (fresh). Similar to the honeydew melon tissue, other spinach subsamples were either bagged and put into a −80 °C freezer (frozen), flash-frozen in liquid nitrogen and then placed into −80 °C (frozen + N_2), lyophilized in plastic bags for 2 days after freezing to −80 °C (Freeze-Dryer 5, Labconco, Kanasas City, MO) (freeze-dried), or acetone-precipitated (acetone ppt). Acetone precipitation was performed as described above. Tissues were either analyzed immediately following preparation or stored at −80 °C for 2, 4, and 8 weeks prior to analysis.

Antioxidant Enzyme Extraction and Assay. For AsPX (EC 1.11.1.11), CAT (EC 1.11.1.6), GR (EC 1.6.4.2), and POX (EC 1.11.1.7), ∼10 g of fresh weight (FW) of both melon mesocarp and spinach leaf tissues was homogenized using a mortar and pestle in 30 mL of ice-cold 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ascorbic acid (added fresh), 1 mM EDTA, 0.5 g of PVP), and ∼2 g of washed and ignited sea sand. For DHAR (EC 1.8.5.4), MDHAR (EC 1.6.5.4), and SOD (EC 1.15.1.1) melon mesocarp and spinach leaf tissues were extracted as above with the exception that ascorbate was absent from the extraction buffer.

The homogenate was centrifuged at 10000g_n for 15 min at 4 °C, and the supernatant was either immediately used for AsPX, CAT, GR, MDHAR, and POX determinations or first passed through a PD-10 Sephadex G-25 column (Pharmacia Biotech, Uppsala, Sweden) before analyses for activities of DHAR and SOD. All steps in the preparation were carried out at 4 °C. Total AsPX, CAT, DHAR, GR, MDHAR, and SOD activities were measured according to the method of Hodges and Forney (*3*), whereas POX activity was measured according to the method of Lamikanra and Watson (*5*), after having been modified for appropriate reaction buffer pH, volume, and enzyme protein concentration.

Enzyme activities were assessed spectrophotometrically at 25 °C. All honeydew melon assays were analyzed on a Perkin-Elmer Lambda 2 UV−VIS spectrophotometer (Perkin-Elmer, Uberlingen, Germany) equipped with a Haake FE-2 water bath (Haake Instruments, Berlin, Germany) for temperature control. All spinach assays were performed on an Ultraspec 3000 spectrophotometer (Pharmacia Biotech) equipped with an Endocal RTE-5B water bath (Neslab, Portsmouth, NH) for temperature control.

AsPX activity was assayed in the presence of 90 mM potassium phosphate buffer (pH 6.0 for melon, pH 7.0 for spinach), 0.1 mM EDTA, 0.65 mM ascorbic acid, and enzyme extract. The assay was initiated with the addition of 1.0 mM H_2O_2. Activity was determined by following the oxidation of ascorbate at 290 nm.

CAT activities were determined in the presence of 100 mM potassium phosphate buffer (pH 6.0 for melon, pH 7.5 for spinach) and enzyme extract. The reaction was initiated with the addition of 50 mM H_2O_2. Activity was determined by monitoring the decomposition of H_2O_2 at 240 nm.

DHAR activity was assessed in the presence of 90 mM potassium phosphate buffer (pH 7.0), 0.97 mM EDTA, 5.0 mM GSH, and either extract or the equivalent volume of extraction buffer for the blank. The reaction was initiated with the addition of freshly made 0.2 mM dehydroascorbate (DHA). Activity was determined by following the reduction of DHA at 265 nm after the nonenzymic reduction of DHA by GSH had been accounted for.

GR activity was determined in the presence of 80 mM Tris-HCl buffer (pH 6.0 for melon, pH 8.5 for spinach), 2.5 mM GSSG, 1.5 mM EDTA, and enzyme extract. The reaction was initiated with the addition of 0.5 mM NADPH in 1% (v/v) $NaHCO_3$ and was followed by monitoring the oxidation of NADPH at 340 nm.

MDHAR activity was assayed in the presence of 90 mM potassium phosphate buffer (pH 6.0 for melon, pH 7.5 for spinach), 0.0125% Triton X-100, 0.2 mM NADH, 2.5 mM ascorbic acid, and enzyme extract. The reaction was initiated with the addition of 5.0 units of ascorbate oxidase. Activity was determined by monitoring the oxidation of NADH at 340 nm.

POX activity was determined in the presence of 100 mM potassium phosphate buffer (pH 6.0 for melon, pH 6.5 for spinach), 10 mM guaiacol, and enzyme extract. The reaction was initiated with the addition of 10 mM H_2O_2, and activity was determined by following the formation of tetraguaiacol at 470 nm.

SOD activity was determined in the presence of 65.0 mM potassium phosphate buffer (pH 7.5), 0.01 mM EDTA, 0.5 mM xanthine, 0.13 mM cytochrome *c*, 0.025 units of xanthine oxidase, and either enzyme or an equal volume of extraction buffer for the blank. Activity was determined by monitoring at 550 nm the inhibition of the reduction of rate of cytochrome *c* between reaction mixtures with and without the protein extract.

Protein Determination. All protein concentrations were determined spectrophotometrically at 595 nm using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA) in a method based on that of Bradford (*9*). Bovine γ-globulin (0.25−1.4 $mg \cdot mL^{-1}$) was used as a standard reference. Although material for the enzyme assays was prepared with a mortar and pestle as described above, tissues were also ground using a Polytron model PT 10/35 homogenizer (Brinkman Instruments Inc., Westbury, NY) or an Osterizer blender (Oster, Sunbeam Products Inc., Maitland, FL) for comparison with the mortar and pestle procedure for enzyme extraction efficiency. No significant differences (data not shown) were found among the three extraction procedures for total extractable protein content or for specific enzyme activity (units of activity per milligram of protein).

Statistical Analyses. Type 1 analysis of variance was used to evaluate enzyme activities, and significant differences were determined by Duncan's multiple-range test for honeydew and spinach tissues (SAS Institute, Cary, NC). Data are expressed as three subsamples/enzyme assay ($n = 3$) per set. Orthogonal comparisons for linear regression (correlation) were determined for enzyme activity by sampling time (SAS Institute).

RESULTS AND DISCUSSION

Prior to storage at −80 °C for 2−8 weeks (prestorage), analyses of variance of honeydew melon fruit and spinach leaf

Table 1. Prestorage Comparison of Sampling Time (Set, S) and Treatment (Tmt, T) Significance of Variability (ANOVA) and Correlation (*r*) of Enzyme Activity by Set for Enzyme Activity in Honeydew Melon Fruit and Spinach Leaves

ANOVA	df	AsPX	CAT	DHAR	GR	MDHAR	POX	SOD
				Honeydew				
S	1	0.0001	0.0001	0.0028	0.3594	0.0080	0.0001	0.0006
T	4	0.0001	0.0040	0.0618	0.0078	0.0001	0.0001	0.2559
S × T	4	0.0001	0.1680	0.0473	0.2561	0.1280	0.5120	0.3886
r		0.93	0.83	0.63	0.56	0.89	0.94	0.57
P		0.0001	0.0001	0.0006	0.0254	0.0001	0.0001	0.02
				Spinach				
S	1	0.0001	0.0001	0.0049	0.3592	0.0001	0.0001	0.0001
T	4	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
S × T	4	0.0001	0.0001	0.7418	0.0018	0.0003	0.0001	0.0001
r		0.97	0.96	0.89	0.94	0.97	0.97	0.96
P		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

antioxidant enzyme activities (AsPX, CAT, DHAR, GR, MDHAR, POX, and SOD) by sampling time (sets) and tissue treatments demonstrated that sets and treatments each had a significant effect (**Table 1**), indicating that even though the different treatments resulted in dissimilar enzyme activities, so did differences in commercial purchase date (honeydew fruit) and maturation (spinach leaves). Moreover, the interaction of set by treatment also produced an effect. For six of seven enzymes from spinach (DHAR being the exception) the interaction was highly significant ($P \geq 0.001$), but the exact opposite results were found for melon, for which six of seven enzymes (AsPX being the exception) were not highly significant for the interaction. This interaction measures the orders of magnitude difference between each of the treatments in one set and compares that to the orders of magnitude difference between each of the treatments in the other set. Differences found in this interaction can be due to differences in tissue sampling size or in nonuniformity of tissue physiological stage (*10*). Honeydew melon fruit enzyme activity, although exhibiting a significant difference for set for almost all enzymes, was not found to be significant for set by treatment interaction. This difference may be explained by the fact that no fewer than five fruits of similar physiological stage were combined and used as the source material for all of the treatments. Spinach, demonstrating significance differences for both set and set by treatment interaction, were not of the same physiological stage (set 1 was harvested after 73 days of growth versus set 2 harvested after 42 days of growth). Although actual enzyme activities from both sets for honeydew and spinach were different, a correlation (*r*) of the enzyme activity profiles for each treatment from each set was statistically the same (**Table 1**), demonstrating that the treatment effects from one set to the next, for each antioxidant enzyme, were very reproducible. This also suggests that, overall, the enzyme assays used in this study were extremely sensitive and highly appropriate for detecting possible treatment differences.

The effect of tissue treatment (frozen to −80 °C, flash-freezing in liquid N_2, freeze-drying, or acetone ppt) on the activities of the seven enzymes during 8 weeks of storage at −80 °C was determined for both melon fruit and spinach leaves (**Tables 2** and **3**). Comparison of both sets of melon fruit indicated that there was no significant change in enzyme activities among tissue frozen to −80 °C, tissue frozen in liquid N_2, or fresh tissue (day 0). However, freeze-drying the tissue or acetone ppt of the proteins affected the activity of all of the enzymes; the activity was either significantly lower or higher than that of the fresh tissue. It should be noted that protein precipitation with ethanol—a common alternative to acetone—may give different results. In general, however, the effect on enzyme activity, either higher or lower, by freeze-drying the tissue or acetone ppt of the proteins was specific for each enzyme and was reproducible from one set to the other. Comparison of enzyme activities between melon set 1 and set

Table 2. Prestorage Activities[a] of the Antioxidant Enzymes AsPX, CAT, DHAR, GR, MDHAR, POX, and SOD in Fresh Honeydew Mesocarp Tissue, in Honeydew Tissue Frozen either to −80 °C (Frozen) or with Liquid Nitrogen (Frozen + N_2), in Freeze-Dried Honeydew Tissue, or as an Acetone Powder Precipitate (Acetone ppt) of Honeydew Tissue (Data Are Means of $n = 3$)[b]

tissue	AsPX	CAT	DHAR	GR	MDHAR	POX	SOD
			Set 1: Honeydew Fruit Purchased Feb 4, 2003				
fresh	9.82a	0.006a	0.29c	0.09a	0.15a	0.54b	0.023a
frozen	8.94a	0.006a	0.28c	0.08a	0.16a	0.64b	0.030a
frozen + N_2	9.35a	0.007a	0.29c	0.07ab	0.16a	0.63b	0.023a
freeze-dried	5.42b	0.002b	0.58a	0.04c	0.14a	0.29c	0.013b
acetone ppt	2.44c	0.008a	0.46b	0.06bc	0.04b	1.01a	0.024a
$P \leq 0.05$							
			Set 2: Honeydew Fruit Purchased Feb 11, 2003				
fresh	6.54a	0.023a	0.52b	0.06a	0.18a	0.84b	0.037a
frozen	8.94a	0.021a	0.56b	0.07a	0.19a	0.85b	0.036a
frozen + N_2	6.17a	0.022a	0.56b	0.05ab	0.19a	0.84b	0.034a
freeze-dried	4.56b	0.006b	0.83a	0.03c	0.18a	0.53c	0.033a
acetone ppt	2.18c	0.022a	0.42c	0.07a	0.07b	1.27a	0.029a
$P \leq 0.05$							

[a] Units for activities are as follows: AsPX, millimolar ascorbate oxidized per minute per milligram of protein; CAT, millimolar H_2O_2 decomposed per minute per milligram of protein; DHAR, millimolar ascorbate reduced per minute per milligram of protein; GR, millimolar NADPH oxidized per minute per milligram of protein; MDHAR, millimolar NADH oxidized per minute per milligram of protein; POX, millimolar tetraguaiacol formed per minute per milligram of protein; and SOD, millimolar cytochrome *c* conserved per minute per milligram of protein. [b] Means within a column followed by the same letter are not significantly different by Duncan's multiple-range test.

Table 3. Prestorage Activities[a] of the Antioxidant Enzymes AsPX, CAT, DHAR, GR, MDHAR, POX, and SOD in Fresh Spinach Leaves, in Spinach Leaves Frozen either to −80 °C (Frozen) or with Liquid Nitrogen (Frozen + N_2), in Freeze-Dried Spinach Leaves, or as an Acetone Powder Precipitate (Acetone ppt) of Spinach Leaves (Data Are Means of $n = 3$)[b]

tissue	AsPX	CAT	DHAR	GR	MDHAR	POX	SOD
			Set 1: Spinach Leaves Harvested after 73 Days of Growth				
fresh	0.94a	0.22b	0.458a	0.10b	0.11b	1.67b	0.04c
frozen	0.82a	0.23b	0.300b	0.11b	0.14a	1.56b	0.04c
frozen + N_2	0.44b	0.27b	0.404b	0.12b	0.14a	2.04b	0.04c
freeze-dried	0.91a	0.38b	0.429ab	0.11b	0.16a	1.52b	0.11a
acetone ppt	0.16c	1.31a	0.003c	0.36a	0.04b	4.99a	0.08b
$P \leq 0.05$							
			Set 2: Spinach Leaves Harvested after 42 Days of Growth				
fresh	0.52ab	0.16b	0.53a	0.13b	0.11a	0.06b	0.02a
frozen	0.53a	0.16b	0.38b	0.12bc	0.10a	0.06b	0.01b
frozen + N_2	0.57a	0.19b	0.46ab	0.11c	0.10a	0.07b	0.01b
freeze-dried	0.48b	0.20b	0.47ab	0.13b	0.11a	0.06b	0.02a
acetone ppt	0.20c	0.29a	0.14c	0.26a	0.02b	0.14a	0.02a
$P \leq 0.05$							

[a] Units of activities are as follows: AsPX, millimolar ascorbate oxidized per minute per millgram of protein; CAT, millimolar H_2O_2 decomposed per minute per milligram of protein; DHAR, millimolar ascorbate reduced per minute per milligram of protein; GR, millimolar NADPH oxidized per minute per milligram of protein; MDHAR, millimolar NADH oxidized per minute per milligram of protein; POX, millimolar tetraguaiacol formed per minute per milligram of protein; and SOD, millimolar cytochrome *c* conserved per minute per milligram of protein. [b] Means within a column followed by the same letter are not significantly different by Duncan's multiple-range test.

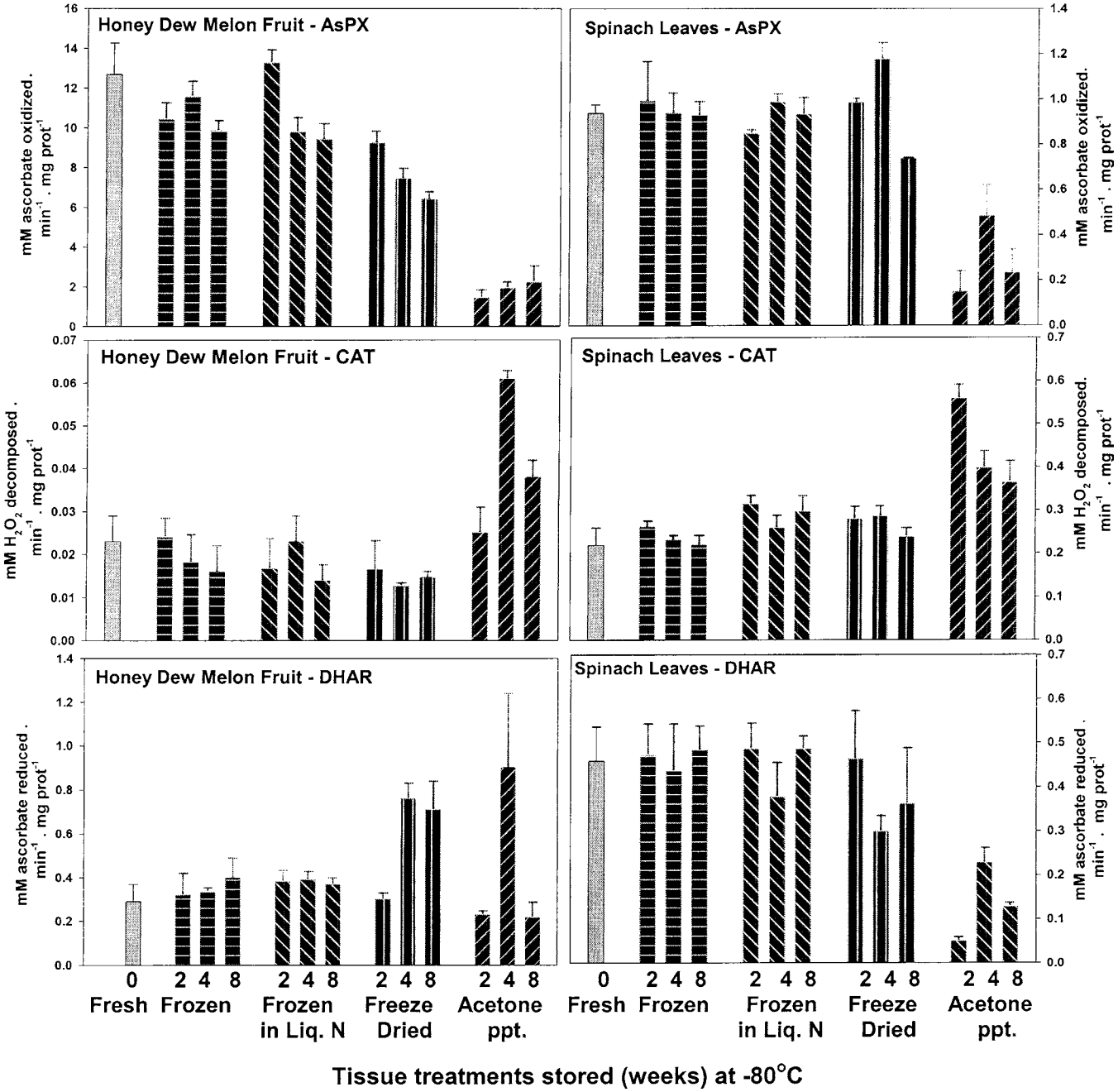

Figure 1. Poststorage changes in honeydew melon fruit and spinach leaf AsPX, CAT, and DHAR enzyme activities in fresh or frozen, frozen in liquid N_2, or freeze-dried tissue or an acetone protein ppt, all stored for 2, 4, or 8 weeks at −80 °C. Bars reflect means ± standard deviations ($n = 3$).

2 demonstrated a slightly higher overall level of activity for set 1. This suggests that, not surprisingly, the genotype and/or production conditions (*11*−*13*) or the commercial handling/storage conditions (*14*) of the fruit may have differed.

There was little affect on the activity of the majority (CAT, GR, MDHAR, and POX) of the enzymes from spinach tissue that were either frozen at −80 °C, frozen in liquid N_2, or freeze-dried compared with activities from fresh material across sets. However, precipitation as an acetone powder affected almost all (AsPX, CAT, DHAR, GR, MDHAR, and POX) activities, generally appearing as an increase. When spinach set 1 (73-day-old plants) and set 2 (42-day-old plants) were compared, the younger leaves (set 2) exhibited lower activity levels for most of the enzymes assayed (AsPX, CAT, MDHAR, POX, and SOD). This suggests that antioxidant enzyme activities will increase with growth and maturation, which corroborates the results of previous research with apples (*15*) and tomatoes (*16*, *17*).

Comparison of enzyme activities following 2, 4, or 8 weeks of storage demonstrated that activities, depending on prestorage treatment, can remain unchanged, increase, or decrease (**Figures 1** and **2**; **Table 4**). In general, for both honeydew melon and spinach, once frozen with or without liquid N_2, there was little significant change in enzyme activities between 2 and 8 weeks at −80 °C. Freeze-dried and acetone ppt tissue treatments prior to storage, however, significantly affected enzyme activities throughout the storage regime. The dehydrated tissue from freeze-drying and acetone precipitation is likely a cause for extreme variability in enzyme activities compared to non-dehydrated treatments (frozen with or without liquid N_2). It has been observed that enzymatic activity is conserved if frozen in a hydrated state and surrounded by cryoprotective substances such

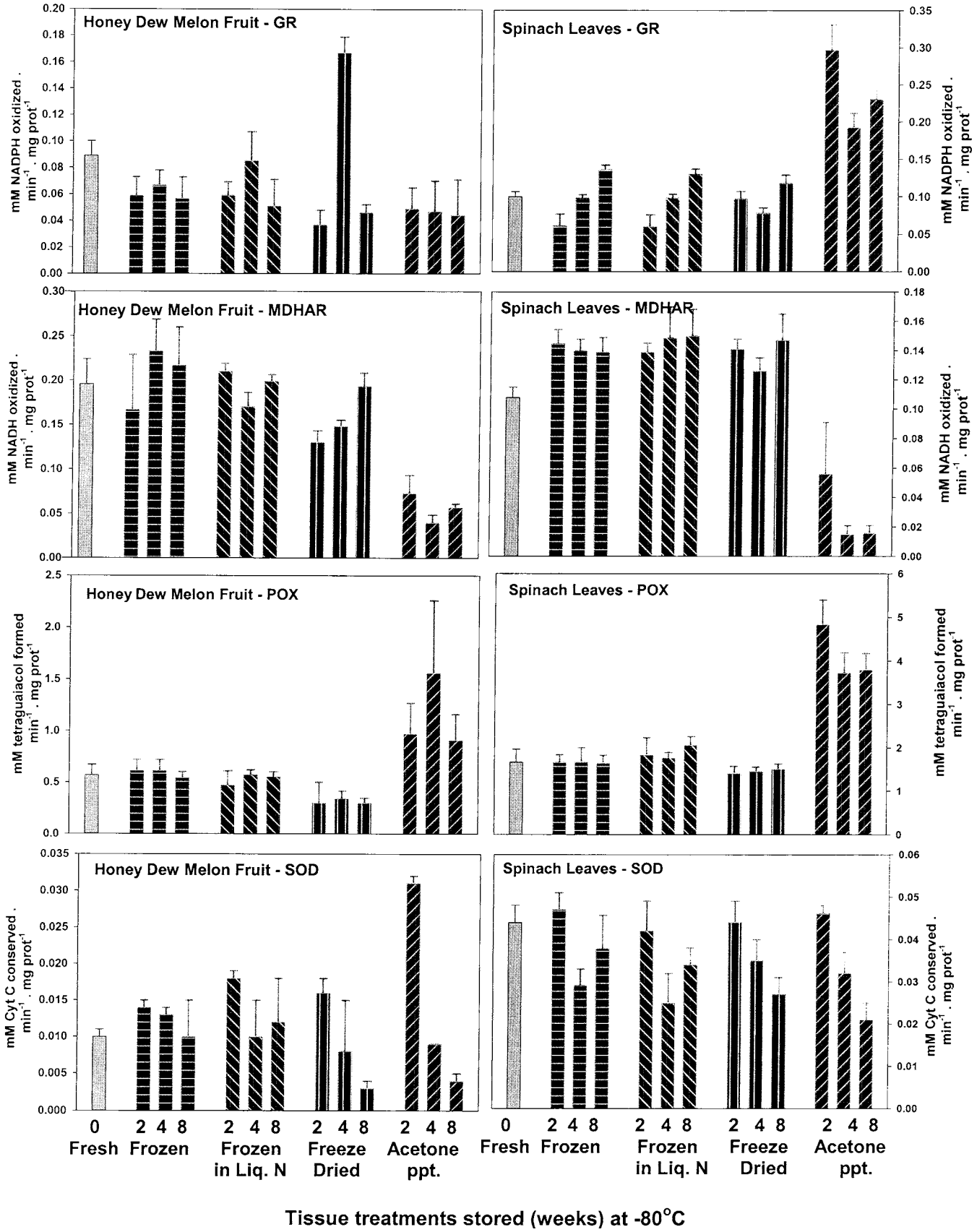

Figure 2. Poststorage changes in honeydew melon fruit and spinach leaf GR, MHDAR, POX, and SOD enzyme activities in fresh or frozen, frozen in liquid N_2, or freeze-dried tissue or an acetone protein ppt, all stored for 2, 4, or 8 weeks at −80 °C. Bars reflect means ± standard deviations ($n = 3$).

as low molecular weight saccharides, that is, sucrose and fructose (*18*, *19*). The cryoprotective sugars sucrose and fructose are found in relatively high concentrations in both honeydew melon fruit (*11*) and spinach leaves (*20*). Alternatively, enzyme activity decreases may have occurred due to denaturation and/or conformational changes related, directly or indirectly, to

Table 4. Poststorage Comparison of Sampling Time (Set, S), Treatment (Tmt, T), and Storage (Weeks, W) Significance of Variability (ANOVA) and Correlation (*r*) of Enzyme Activity by Set for Enzyme Activity in Honeydew Melon Fruit and Spinach Leaves

ANOVA	df	AsPX	CAT	DHAR	GR	MDHAR	POX	SOD
				Honeydew				
S	1	0.0051	0.0001	0.0002	0.0334	0.0372	0.0030	0.2850
T	3	0.0001	0.0001	0.0001	0.3974	0.0001	0.0001	0.0588
W	2	0.0001	0.0174	0.0001	0.0001	0.0022	0.7843	0.0004
S × T	3	0.0001	0.0480	0.0100	0.0170	0.1848	0.2229	0.9879
S × W	2	0.0531	0.0890	0.0511	0.0631	0.0197	0.0370	0.0276
T × W	6	0.0020	0.0241	0.0288	0.0329	0.0258	0.0148	0.3567
S × T × W	6	0.1211	0.1284	0.0110	0.1171	0.5611	0.0331	0.1368
r		0.95	0.90	0.93	0.79	0.84	0.83	0.51
P		0.0001	0.0001	0.0001	0.00001	0.0001	0.0001	0.01
				Spinach				
S	1	0.0001	0.0001	0.1328	0.4430	0.0001	0.0001	0.0001
T	3	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
W	2	0.0253	0.0001	0.0012	0.0001	0.3038	0.0003	0.0001
S × T	3	0.0001	0.0011	0.0082	0.0004	0.4202	0.0001	0.3725
S × W	2	0.0002	0.0001	0.0002	0.2915	0.0818	0.2275	0.0001
T × W	6	0.0001	0.2654	0.1189	0.0001	0.3639	0.0931	0.0578
S × T × W	6	0.4339	0.0184	0.2967	0.0121	0.1151	0.0891	0.0823
r		0.93	0.97	0.91	0.95	0.85	0.98	0.92
P		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

prolonged exposure to low temperatures of < −80 °C. It is possible that the process of freeze-drying and/or acetone precipitation may have fractured organelles and membranes over and above mortar and pestle homogenization, releasing more enzyme and contributing to the observed increases in enzymic activities. Often, such as in the case of GR, enzyme activities increased in freeze-dried and/or acetone-precipitated material in one of the species tested but not the other.

CONCLUSIONS

Sanchez-Moreno (*21*), in a review of methods to evaluate the free radical scavenging (antioxidant) activity in plant tissues, laments that due to the diversity of methods to determine antioxidant activity, research is greatly needed to standardize these measurements. We agree and add that standardizing the pre-extraction preparation procedures of antioxidant-containing tissues is equally critical. Although extraction of antioxidant enzymes from tissues follows well-established procedures, pre-extraction preparation of tissues for storage does not. Our data show that fresh versus frozen to −80 °C or flash-frozen in liquid N_2 results in no significant change in the activities of seven of the most important plant antioxidant enzymes (AsPX, CAT, DHAR, GR, MDHAR, POX, and SOD). However, pre-extraction treatments, whereby the tissue was dehydrated during either freeze-drying or acetone powder precipitation, usually caused either significantly lower or higher enzyme activities when compared with those from fresh tissue.

Using a representative fruit (honeydew melon) and vegetable (spinach leaves) we have demonstrated that the type of tissue treatment prior to −80 °C storage and subsequent enzyme extraction was highly critical and drastically altered enzyme activity levels. From the results of this study, we suggest that the standard procedure for storage of plant tissues for antioxidant enzyme activities should involve a protocol, whereby the tissues are maintained in a hydrated state, either frozen to −80 °C or flash-frozen in liquid N_2.

ABBREVIATIONS USED

AsPX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbic acid; DHAR, dehydroascorbate reductase; FW, fresh weight; GR, glutathione reductase; GSH, glutathione reduced; GSSG, glutathione oxidized; MDHAR, monodehydroascorbate reductase; POX, guaiacol peroxidase; SOD, superoxide dismutase.

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